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RAPID COMMUNICATION

The Interferon-Inducible 204 Gene Is Transcriptionally Activated by Mouse Cytomegalovirus and Is Required for Its Replication

Sandra Rolle,*[,1] Marco De Andrea,*[,†,1] Daniela Gioia,† David Lembo,† Laura Hertel,*[,†] Santo Landolfo,*[,‡] and Marisa Gariglio†[,2]

*Department of Public Health and Microbiology, Medical School of Torino, Turin, Italy; †Department of Medical Sciences, Medical School of Novara, Novara, Italy; and ‡Immunogenetics and Experimental Oncology Center, C.N.R., Turin, Italy

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Infection of cells with viable or UV-inactivated murine cytomegalovirus (MCMV) increased the IFN-inducible 204 gene at both the mRNA and the protein levels. The activity of a reporter gene driven by the mouse Ifi204 promoter induced following virus infection showed that this increase was due to transcriptional activation. Moreover, FACS analysis of infected mouse embryo fibroblasts (MEF) stably transfected with a p204-dominant-negative mutant (p204dmMEF) revealed that they do not accumulate at the G1/S border in the same way as infected MEF transfected with the empty vector (neoMEF). MCMV DNA synthesis is significantly delayed (144 h in p204dmMEF vs 72 h in neoMEF), due to retarded expression of viral genes, namely, IE1 and DNA polymerase, as shown by Western blot comparison of p204dmMEF and neoMEF extracts. These results demonstrate that MCMV may exploit the Ifi204 gene to regulate the cell cycle and enhance its DNA synthesis. © 2001 Academic Press

Introduction, results, and discussion. Infection of permissive cells with cytomegalovirus (CMV), a β-herpesvirus, results in the progression of a sequentially ordered set of physiological responses similar to growth factor- or cytokine-induced cellular activation (*1, 3*). However, this activation does not lead to cell division (*14*). Earlier studies revealed indeed that CMV rapidly inhibits the growth of fibroblasts, by blocking cell cycle progression at multiple points, including the G1-to-S-phase transition (*3, 6, 7, 18*). The demonstration by Salvant *et al.* (*20*) that infection in the S phase delays the expression of immediate-early (IE) viral genes further emphasizes the importance of cell cycle regulation for human cytomegalovirus (HCMV) replication. However, in contrast to this blockade, CMV infection also stimulates the growth-regulated pathway together with a number of enzymes involved in nucleotide metabolism and DNA replication (*10, 16*).

Differential display analysis and DNA microarray assay demonstrated that HCMV infection before the onset of virus DNA synthesis caused changes of about 258 cellular mRNAs, some of which are also induced by interferons (IFNs) (*21*). In line with these findings it has been demonstrated that soon after infection HCMV activates interferon immediate-early response gene expression and interferon regulatory factor-3 (IRF-3) containing an interferon-stimulated response element-binding complex (*19*). The principal mechanism by which CMV induces intracellular signaling and activation of the interferon response is via an interaction of the primary viral ligand, glycoprotein B (gB), with its cognate cellular receptor, although other mechanisms involving different receptors and signal transduction pathways cannot be excluded (*2*).

Among the IFN-stimulated genes (ISGs), one family, designated Ifi200, includes the p202, p203, p204, and D3 proteins and their human homologues, myeloid nuclear differentiation antigen, Ifi16, and AIM2 (*15*). The 204 protein, encoded by the Ifi204 member of the Ifi200 gene cluster, contains two LXCXE motifs that are potential sites for binding to the retinoblastoma (Rb) gene product and functions as a growth suppressor in sensitive cell lines by delaying G0/G1 progression into S phase (*13, 17*) and impairing E2F-mediated transcriptional activity (*11*). Recent results from our laboratory demonstrated that expression of mutant 204 proteins devoid of one or both domains interferes with the murine cytomegalovirus (MCMV) multiplication cycle, but does not affect IFN antiviral activity, suggesting that the IFN-inducible protein 204 is somehow related to MCMV replication (*12*). The finding that inactivation of p204 by dominant-negative mutants deregulates the cell cycle and renders cells intrinsically resistant to MCMV replication might there-

[1] S. Rolle and M. De Andrea contributed equally to this work.

[2] To whom correspondence and reprint requests should be addressed at Department of Medical Sciences, Medical School of Novara, Via Solaroli, 17, 28100 Novara, Italy. Fax: +39.0321.620421. E-mail: gariglio@med.unipmn.it.

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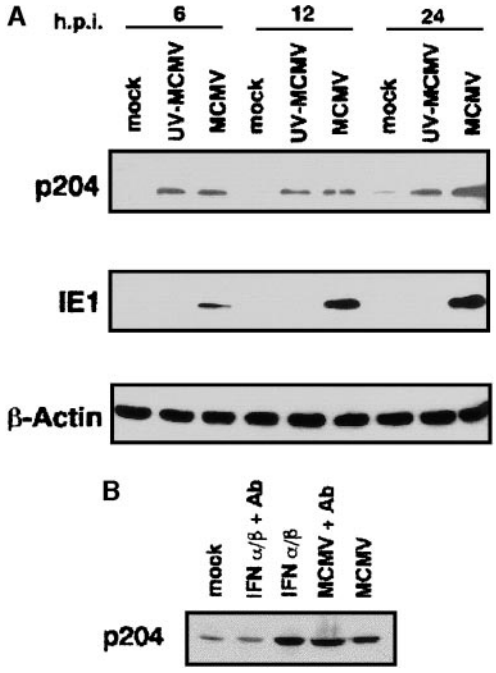

FIG. 1. (A) Induction of p204 during MCMV infection. MEF cultured at 2% FBS were infected with MCMV (m.o.i. of 2), infected with UV-irradiated MCMV, or mock-infected. Total cell extracts were prepared at the indicated time points after infection, fractionated by SDS–PAGE (30 μg/lane), and analyzed by immunoblotting with anti-p204 or anti-IE1 Abs. The membrane was then incubated with goat anti-rabbit Ig horseradish peroxidase conjugate as secondary antibody and visualized with an ECL kit (Amersham). Actin immunodetection was performed as an internal control. (B) Anti-IFN-α/β Abs do not block p204 induction by MCMV. MEF cultured at 2% FBS were incubated with 100 neutralization units of sheep polyclonal Abs against mouse IFN-α/β for 1 h and then infected with MCMV (m.o.i. of 2). As a control, 100 U of mouse IFN-α/β was added with or without preincubation with 100 neutralization units of anti-IFN Abs to MEF cultured at 2% FBS. After 24 h total cell extracts were prepared and analyzed by immunoblotting with anti-p204 Abs.

fore suggest that MCMV exploits p204 to regulate the cell cycle and consequently its DNA synthesis.

To determine whether MCMV infection up-regulates p204 expression, lysates from MCMV-infected mouse embryo fibroblasts (MEF) were assessed for its expression by Western blotting analysis. Time course experiments revealed a detectable induction of p204 levels as early as 6 h postinfection (p.i.). This gradually increased at 12 h p.i., peaked at 24 h p.i., and then declined (Fig. 1A). To see whether this induction was due to virion constituents or viral gene expression, cells were also infected with UV-inactivated MCMV and expression of viral IE1 was used as a marker of infectivity. As shown in Fig. 1A, p204 induction does not appear to require viable MCMV virions, since infection of MEF with the UV-inactivated virus resulted in its up-regulation in a manner comparable to that for replication-component virus. To confirm that UV-inactivated virus does not express IE genes, cell extracts were also probed with anti-IE1 antibodies (Abs). As shown in Fig. 1A, UV inactivation blocked IE gene expression without affecting p204 induction. As expected, mouse IFN-α/β induced p204 at levels slightly higher than those observed with MCMV (data not shown).

IFN-α/β was not detectable in the supernatants from infected MEF positive for p204 expression, excluding the possibility that it was triggered by CMV through induction of IFN (data not shown). Moreover, addition of Abs against mouse IFN strongly affected p204 induction upon IFN treatment, but did not impair its activation upon MCMV infection (Fig. 1B), demonstrating that MCMV directly stimulates p204 expression.

To determine whether the increase in p204 levels observed in MCMV-infected MEF represented specific activation of its transcription, MCMV-infected and IFN-treated fibroblasts were first analyzed by semiquantitative RT-PCR. Time course analysis demonstrated that stimulation of cells with either MCMV or IFN-α resulted in the rapid induction of Ifi204 mRNA levels (Fig. 2A). The abundance of Ifi204 mRNA was significantly up-regulated (fivefold) compared with the untreated control. Levels for the MCMV-infected cells peaked after 3 h of stimulation and then declined. As observed in Western blotting analysis, UV inactivation did not impair Ifi204 stimulation, confirming that virion constituents are responsible for gene induction (Fig. 2A). The activation response to IFN treatment was very similar (sixfold), except that the induction lingered longer and was still detectable 12 h p.i.

Ifi204 expression is regulated primarily at the transcriptional level following IFN treatment (*15*). The effects of MCMV infection on the expression of a transiently transfected luciferase reporter gene driven by the Ifi204 promoter were therefore evaluated. MEF were transfected by lipofectamine with the reporter plasmid pLuc204 (which contains the luciferase cDNA driven by the Ifi204 promoter) and then infected with MCMV at a m.o.i. of 2 for 6 or 18 h. As shown in Fig. 2B, MCMV infection resulted in Ifi204 promoter stimulation that began at 6 h p.i., as indicated by the significant increase of the luciferase activity (about threefold induction), and reached a maximum at 18 h p.i. (about sixfold induction). In accord with the Western blotting analysis, the activation potentials of viable and UV-inactivated MCMV were equivalent, indicating that MCMV-mediated transactivation is generated by interaction of virion constituents with the cell membrane.

The finding that MCMV induces expression of the Ifi204 gene raised the question of whether it is merely a by-product of CMV infection or whether MCMV induces ISGs such as Ifi204 to actually regulate cell cycle progression and create an environment favorable to its replication.

To verify this hypothesis we investigated the impact of CMV infection on the cell growth of neoMEF and p204dmMEF, stably transfected with a p204 mutated at

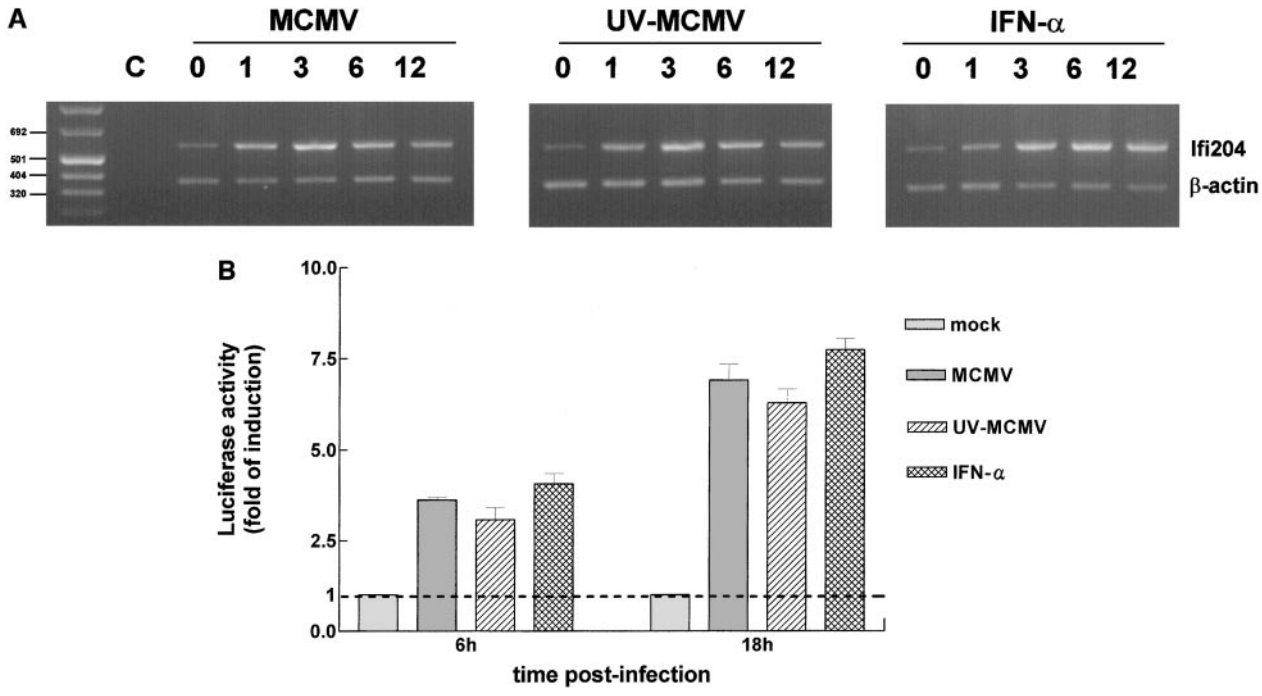

FIG. 2. (A) Induction of Ifi204 mRNA during MCMV infection. MEF cultured at 2% FBS were infected with MCMV (m.o.i. of 2) or UV-irradiated MCMV or treated with IFN-α/β (1000 U/ml). Total RNA was isolated at the indicated time points, retrotranscribed, and analyzed by PCR. Amplification was performed by Ifi204 sense and antisense primers originating a 567-bp fragment. The β-actin (348-bp fragment) was coamplified as an internal control. Data are representative of a single experiment performed independently four times. (B) Effect of MCMV infection of Ifi204 promoter activity. DNA (2 μg) from p204Luc or pGL3 was transiently transfected into MEF as described under Materials and Methods. After 48 h cells were infected with MCMV (m.o.i. of 2) or UV-irradiated MCMV or treated with IFN-α/β (1000 U/ml). Total cytoplasmic extracts were isolated at the indicated time points and assayed for luciferase activity. Reporter gene activity was normalized to the amount of plasmid DNA introduced into recipient cells by DNA dot-blot analysis. The resulting luciferase activity is expressed as the fold induction relative to the basal level measured in cells transfected with p204Luc and then mock-infected, which was set at 1. The data represent the means of the normalized luciferase activities from at least three repeated experiments.

both Rb-binding sites that behaves like a dominant-negative inhibiting accumulation of IFN-treated cells at the G1/S border (*13*). To determine whether accumulation of MCMV-infected cells in the G1 phase of the cell cycle was impaired by overexpression of the dominant-negative p204 mutant, FACS analysis was performed. Both neoMEF and p204dmMEF, plated at 80% confluence, were infected at a m.o.i. of 2 as described previously and maintained in 2% FBS. Cells were then harvested at various times p.i. and the DNA content in isolated nuclei was measured by propidium iodide (PI) staining and FACS analysis. Virus-infected neoMEF displayed a G0/G1 peak (51.9% cells with 2*N* content) at 24 h p.i. (Fig. 3A) that broadened and became progressively more intense at 72 h p.i., consistent with the expected accumulation of viral DNA. Mock-infected neoMEF, cultured at 2% FBS, exhibited a G0/G1 peak of 49.4% cells at 24 h after mock infection that increased progressively to 81.2% at 120 h. By contrast, the DNA profile of the infected p204dmMEF was significantly different (Fig. 3B). At 24 h p.i. approximately 35.0 and 47.6% of these MEF were in G0/G1 and S phase, respectively. These cells continued to cycle normally and reached confluence at 96 h p.i., with only 14.4% of cells being in S phase. However, at 120 h p.i. the G0/G1 peak started to broaden and reached the highest intensity at 144 h p.i., compared with 72 h p.i. for the neoMEF. To exclude the possibility that the amount of viral DNA could be moving cells from 2*N* to greater than 2*N*, gancyclovir (GCV) was added at the beginning of the infection to remove the obscuring effects of viral DNA replication. As shown in Fig. 3C, in the presence of GCV, both neoMEF and p204dmMEF at 72 and 120 h p.i. accumulated at the G1/S border, demonstrating that broadening of the cell profile with a content greater than 2*N* was due to viral DNA synthesis.

In view of these results, it was important to determine whether the delay in the onset of viral DNA synthesis was associated with a delay in MCMV protein expression. neoMEF and p204dmMEF were therefore infected as described for FACS analysis, and at the indicated times cell lysates were prepared and analyzed by Western blotting for viral protein expression. As probes, we used Abs specifically recognizing the MCMV IE1 p89 or the E viral protein DNA polymerase (DNApol). As shown in Fig. 4, IE1 protein in infected neoMEF started to increase as early as 6 h p.i. and was highly expressed at 48 h p.i. By contrast, in infected p204dmMEF, IE1 was detectable only at 72 h and was highly expressed at 96 h. The kinetics of the appearance of the viral early protein DNApol was also slower in the p204 mutants. The band

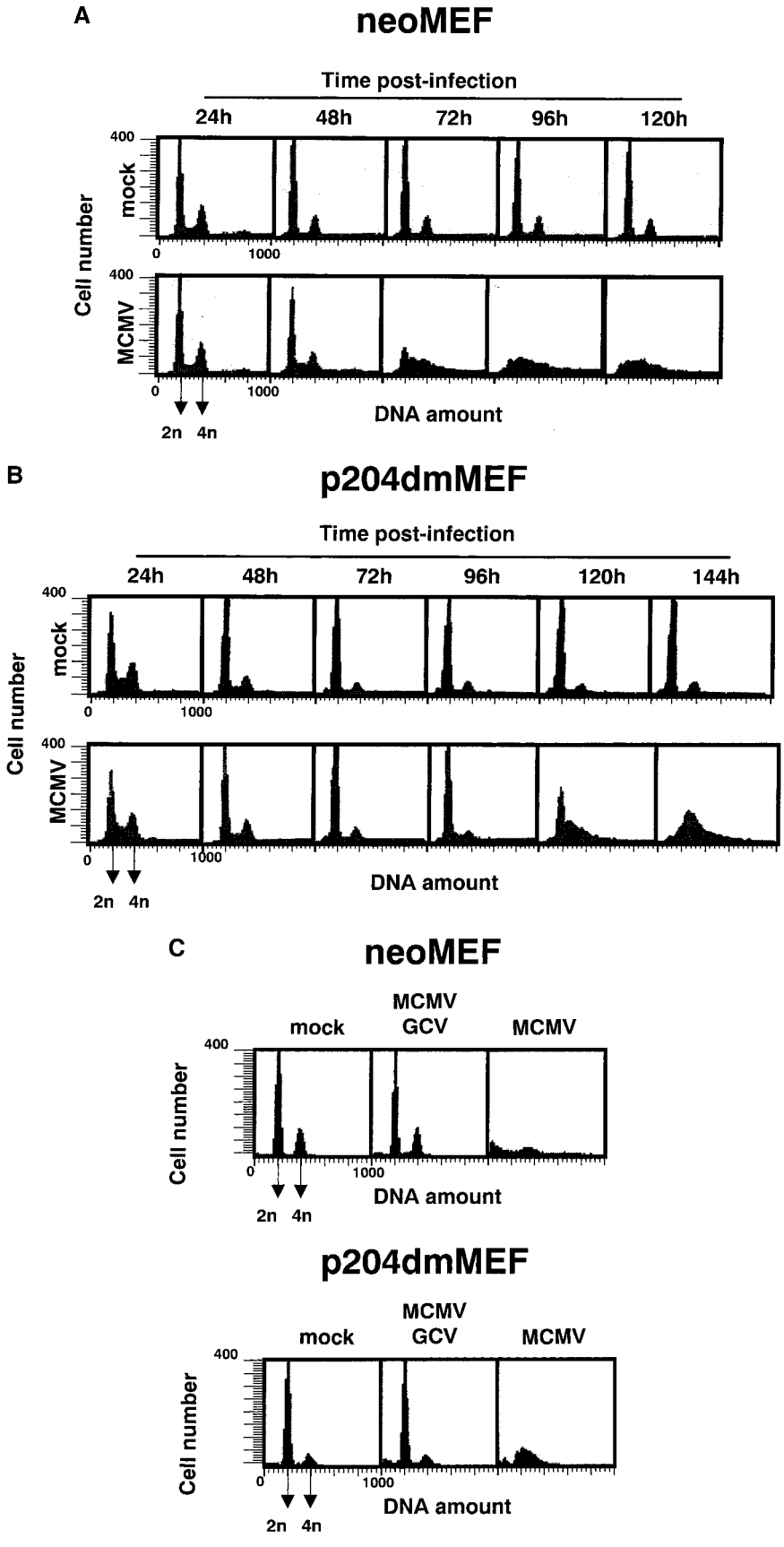

FIG. 3. Inactivation of endogenous p204 delays MCMV DNA synthesis. MEF was transfected with p204dm, encoding the dominant-negative p204 mutated at both Rb-binding sites (p204dmMEF), or the empty vector pRcRSV (neoMEF). Twelve days after transfection, resistant clones were pooled,

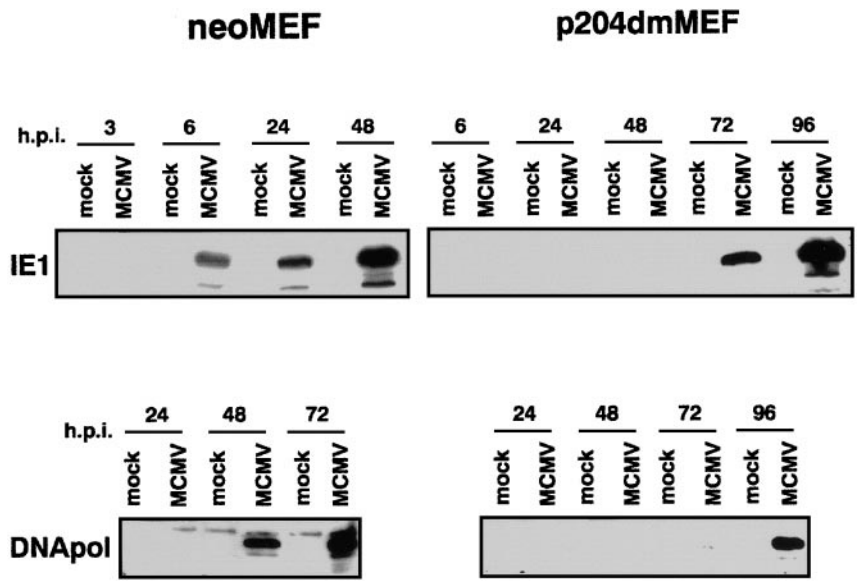

FIG. 4. Expression of viral IE1 and DNA polymerase in p204dmMEF or neoMEF during MCMV infection. p204dmMEF or neoMEF were infected with MCMV (m.o.i. of 2) or UV-irradiated MCMV or mock-infected in 2% FBS. Total cell extracts were prepared at the indicated time points after infection, fractionated with SDS–PAGE (30 μg protein/lane), and analyzed by immunoblotting with anti-IE1 or anti-DNA polymerase Abs. The membrane was then incubated with goat anti-rabbit Ig horseradish peroxidase conjugate as secondary antibody and visualized with an ECL kit (Amersham).

specific for the viral DNApol was not detected at 24 h p.i. in neoMEF, started to increase at 48 h p.i., and peaked at 72 h p.i. A different pattern of protein induction was observed when p204dmMEF were analyzed: a band corresponding to the viral DNApol was barely detectable at 72 h p.i. and was highly expressed at 96 h p.i., correlating the level of protein expression with the kinetics of viral DNA synthesis observed by FACS analysis.

In activating the host cell metabolism to allow optimal viral replication, MCMV infection alters the expression of key cell cycle-regulatory proteins and this ultimately leads to cell cycle arrest (*14, 20*). The Ifi204 gene encodes a protein that controls transition from the G1 to the S phase by interacting with the retinoblastoma and E2F proteins (*13, 15, 17*). Inactivation of the endogenous p204 by dominant-negative mutants bearing a modified Rb-binding motif significantly impairs MCMV replication and delays the appearance of the cytopathic effect (CPE). In this report, we present data that provide insight into the nature of the virus–host interaction that culminates in cell cycle arrest. Binding of MCMV to the cell membrane of normal MEF activates p204 expression. This is accompanied by expression of viral IE genes that then leads to early gene induction and onset of DNA synthesis. It is therefore conceivable that MCMV induces p204 to accumulate infected cells to the G1/S border and create an environment more favorable to its DNA synthesis and replication. This conclusion can be inferred from the results of both FACS analysis and Western blotting in infected p204dmMEF. In the absence of physiological levels of endogenous p204, the onset of IE and E genes and viral DNA synthesis is significantly delayed and retards the appearance of the CPE.

Previous studies have shown that HCMV, probably by engaging the cellular receptor for gB, activates the IRF-3-containing IFN-stimulated response element-binding complex that in turn leads to the expression of two IFN-inducible genes, namely, the OAS and ISG54 (*2, 19*). These studies, however, failed to establish whether the induced genes were required for HCMV replication. Our studies now demonstrate for the first time that members of another family of IFN-inducible genes, the Ifi200 genes, which encode proteins that regulate the progression from G1 to S phase, are stimulated by virion components during the early stage of CMV infection and are required for its replication.

Altogether, these results demonstrate that infection in S phase of rapidly dividing cells, in which the endogenous p204 has been down-regulated by dominant-negatives, delays progression of the viral life cycle and is

plated at 80% confluence, and infected with MCMV at a m.o.i. of 2 in 2% FBS. To prevent MCMV DNA synthesis, 20 μg/ml of GCV was added to the culture after virus adsorption and left for the entire period of infection (72 h for neoMEF and 144 h for p204dmMEF). Cells were then harvested and the DNA content in isolated nuclei was measured by PI staining and FACS analysis. Data are from a single experiment representative of three independent experiments with similar results. (A) Untreated mock-infected or infected neoMEF. (B) Untreated mock-infected or infected p204dmMEF. (C) Gancyclovir-treated neoMEF and p204dmMEF.

associated with a significant fraction of the cells progressing through S phase and G2/M. The intracellular environment during active DNA synthesis may not be favorable for the early stages of viral replication. In the attempt to correlate stimulation of host DNA synthesis by CMV and lack of expression of a subset of an early virus gene, DeMarchi and Kaplan (*5*) proposed that (i) cellular DNA replication and efficient synthesis of viral proteins were mutually exclusive events and (ii) an early gene product expressed in cells fully permissive for the infection was required for this apparent inhibition of cell DNA synthesis (*4*). Consistent with this hypothesis, our data demonstrate that CMV infection dissociates the cell cycle from cell DNA synthesis by early activation of the IFN pathway. This results in the induction of cellular genes (i.e., the Ifi204) capable of inhibiting progression from the G1 to the S phase and allows expression of viral IE genes, which may then sequester and exploit cellular genes of the DNA metabolism, i.e., DHFR, TS, and Rr for viral DNA replication.

Materials and methods. MCMV (mouse salivary gland virus, strain Smith, ATCC VR.194) was purchased from ATCC (Rockville, MD) and produced as described (*16*). Inactivation of virus by UV light was performed as previously described (*16*) and verified by both IE1 expression (Fig. 1) and cytopathic effect. No plaques were observed after 10 days in culture, confirming that the virus was completely inactivated (data not shown).

The plasmid pRcRSV204dmRb (p204dm) was constructed as previously described (*12*). The Ifi204 promoter vector (p204Luc) was constructed by subcloning the Ifi204 promoter region (*Hin*dIII–*Xba*I segment, 1277 bp in length, blunt-ended) from a Lambda FIX II genomic clone into the *Sma*I site of pGL3Basic (Promega) luciferase reporter plasmid.

Mouse embryo fibroblasts 3T3 were kindly provided by T. Upton (Dana-Farber Cancer Institute) and cultured as previously described (*12*). Cells were transfected by lipofectamine and a pooled cell population of neomycin-resistant clones at 12–14 days were designated p204dmMEF when transfected with a vector encoding the p204 mutated at both Rb-binding sites (p204dm) or neoMEF when transfected with the empty vector (pRcRSV, Invitrogen), as previously described (*12*). For transient transfections with p204Luc, MEF were infected with MCMV at a m.o.i. of 2 at 48 h after transfection and at the indicated time points luciferase activity was measured as previously described (*16*).

Rabbit polyclonal monospecific Abs raised against the C-terminal moiety of p204 and the viral IE-1 protein have been previously described (*8, 9*). The presence of the MCMV DNA polymerase was detected with polyclonal monospecific Abs raised by injecting rabbits with the purified GST–DNApol C-terminus (amino acids 791–1031) fusion protein. The sera obtained after the animals were bled 1 week after the fourth immunization were purified on a protein A affinity column (Pharmacia) according to the specifications of the supplier. Mouse IFN-α/β and sheep polyclonal Abs directed against mouse IFN-α/β were from ICN and RDI, respectively. Monoclonal anti-actin Abs (Boehringer) were used as an internal control.

For Western blotting analysis, total cell extracts prepared by lysing in 3% SDS–lysis buffer were separated on 8.5% SDS–polyacrylamide gel and transferred onto a PVDF membrane (Amersham) as previously described (*13*). Total cellular RNA was isolated by using the total RNA extraction solution EUROzol (EUROclone). One microgram of total RNA was reversed-transcribed into mRNA-dependent cDNA by using Moloney murine leukemia virus RT (Ambion Inc.). A 1-μg aliquot for each sample served as a non-RT control for genomic contamination in subsequent PCRs. Equivalent amounts of cDNA were then subjected to PCR analysis performed in a Hybaid PCR Express Thermal cycler. The Ifi204 primers (sense, 5′AAAGAGACAACCAAGAGCAATACACC3′) and antisense, 5′GAATGTTAGATGAAGCCGAAGATGAG3′) amplified a 567-bp fragment. The β-actin primers amplified at 348-bp fragment. For amplification of the DNA fragments, a cycle number in the middle of the linear amplification range (with a minimum of 25 and a maximum of 35 cycles) was used. The reaction mixtures for each sample were added to a tube containing 50 pmol each of the oligonucleotide primers for amplification of both Ifi204 and β-actin. PCR was carried out as follows: a 5-min denaturation step at 95°C followed by 30 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 30 s, followed by 72°C for 5 min to allow for complete extension. Under these conditions, amplification of Ifi204 and β-actin product is in the exponential phase and the assay is linear with respect to the amount of input RNA. Water controls in which all components of the RT-PCR were present, except RNA, were also included. PCR products were electrophoresed on 1.5% agarose and stained with ethidium bromide for visualization. Quantification was performed by scanning the specific bands and measuring the density and the width of each PCR product by using Quantity One software (Bio-Rad Laboratories Inc., Herclues, CA).

For the analysis of DNA content, cells were infected with MCMV at a m.o.i. of 2. At the time of harvest, cells were washed with PBS, counted, and stored at 4°C in PBS with 40% ethanol. DNA content was determined by flow cytometry following staining of the cells with propidium iodide as described previously (*13*).

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